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2D-GEL ELECTROPHORESIS และการประยุกต์ใช้ทางชีวเวชศาสตร์

ชนิตรา ธุวจิตต์

บทคัดย่อ

กว่า 20 ปีมาแล้วที่เทคนิค 2D-gel electrophoresis ถูกพัฒนาขึ้นเพื่อใช้เป็นเครื่องมือในการวิเคราะห์ความซับซ้อนของโปรตีนในเซลล์ เนื้อเยื่อหรือตัวอย่างชีวภาพต่างๆ ภายใต้เทคนิคนี้โปรตีนบนเจลจะถูกแยกใน 2 มิติ โดยอาศัยคุณสมบัติของ isoelectric point (pI) ในมิติที่หนึ่ง และน้ำหนักโมเลกุลในมิติที่สอง เทคนิคนี้มีประสิทธิภาพสูง สามารถจำแนกโปรตีนได้มากถึง 10,000 ชนิด โดยที่แต่ละจุดที่ปรากฏบนเจลคือโปรตีนชนิดเดียว และเมื่อไม่นานมานี้ได้มีการพัฒนาเทคนิค 2D-differential in-gel electrophoresis (2D-DIGE) ขึ้น ซึ่งเทคนิคใหม่นี้สามารถจำแนกโปรตีนจากตัวอย่างที่แตกต่างกันได้ภายในเจลเดียวกัน โดยอาศัยการติดฉลากโปรตีนตัวอย่างด้วยสารเรืองแสงกลุ่มไซยามีนที่มีสีต่างกัน การเปรียบเทียบปริมาณโปรตีนด้วยเทคนิค 2D-DIGE สามารถลดความแปรผันของการทำ 2D-gel electrophoresis แบบเดิม ทำให้สามารถเปรียบเทียบปริมาณที่แตกต่างกันของโปรตีนในตัวอย่างต่างๆ ได้แม่นยำยิ่งขึ้น ในปัจจุบันมีการประยุกต์ใช้เทคนิค 2D-gel electrophoresis และ 2D-DIGE อย่างแพร่หลาย โดยเฉพาะในการวิเคราะห์โปรตีนทั้งหมดที่ปรากฏในเซลล์ (proteomic analysis) ในทางชีวเวชศาสตร์นั้นเทคนิคทั้งสองนี้สามารถประยุกต์ใช้ในการตรวจวัด biomarker และการติดตามการรักษาโรคต่างๆ การพัฒนาวัคซีน การศึกษากลไกการทำงานและพิษวิทยาของยา และการศึกษาทางมะเร็งวิทยา ตัวอย่างการประยุกต์ใช้ ได้แก่ การตรวจพบโปรตีนหลายชนิดที่จำเพาะในเลือดของผู้ป่วยตับอักเสบชนิดบี ในน้ำไขข้อของผู้ป่วยโรครูมาตอยด์ และในชิ้นเนื้อมะเร็งชนิดต่างๆ และการนำเทคนิคนี้ไปใช้ในการตรวจรูปแบบการแสดงออกของโปรตีนในเลือดของผู้ที่ได้รับยาเพื่อบ่งชี้กลไกการทำงานของยา อย่างไรก็ตามแม้ว่าเทคนิค 2D-gel electrophoresis และ 2D-DIGE จะช่วยในการศึกษาการแสดงออกของโปรตีนในเซลล์ ณ ช่วงเวลาหนึ่งได้เป็นอย่างดี แต่ยังมีข้อด้อยในการวิเคราะห์โปรตีนที่แปรผันไปตามเวลาและสิ่งแวดล้อมที่เปลี่ยนไปของเซลล์ ดังนั้นคาดว่าในอนาคตการพัฒนาเทคนิคดังกล่าวให้สามารถศึกษาการแสดงออกของโปรตีนตามช่วงเวลาที่เกิดขึ้นจริง จะช่วยให้การศึกษาหน้าที่ของโปรตีนชนิดนั้นๆ ใกล้เคียงสิ่งที่เกิดขึ้นจริงในเซลล์มากยิ่งขึ้น นำไปสู่การศึกษาพยาธิชีววิทยาของโรค ปรับปรุงการวินิจฉัยและพัฒนาการรักษาโรคต่างๆ ให้มีประสิทธิภาพมากขึ้น

คำหัส: 2D-gel electrophoresis, 2D-DIGE, proteomic, biomedical application

1. INTRODUCTION

Two dimensional (2D)-gel electrophoresis was originated independently but simultaneously by O'Farrell and Klose in 1975.[1,2] O'Farrell used 2D-gel electrophoresis to resolve 1,100 different protein components from *E. coli.*[1] In the same year, Klose tested this technique for a number of

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2D-GEL ELECTROPHORESIS AND ITS BIOMEDICAL APPLICATION

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ABSTRACT

Two dimensional (2D)-gel electrophoresis was originated 20 years ago as a tool for analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. Proteins are separated in gel according to isoelectric point (pI) in the first dimension and molecular weight in the second one. This technique has high capacity that can resolve as many as 10,000 proteins in a gel. Each spot on the 2D-gel represents one type of protein. Recently, the introduction of 2D-differential in-gel electrophoresis (2D-DIGE) as the modified 2D-gel electrophoresis allows different samples tagged with different colored fluorescent cyamine dyes to separate on a single gel. This effectively removes system variation enabling more accurate quantitation of real protein differences between samples than that from conventional 2D-gel electrophoresis. Nowadays, the applications of 2D-gel electrophoresis and 2D-DIGE have rapidly extended worldwide especially in the proteomic analysis for detection of whole protein expression in cell at the certain time. The biomedical applications of 2D-gel electrophoresis and 2D-DIGE, for example, are biomarker detection, monitoring therapies, vaccine development, drug study and cancer research. The examples of these applications include the detection of specific proteins in HBV-infected sera, synovial fluid from rheumatoid patients and a variety of cancer tissues. The application of these 2 techniques is to detect serum proteins in patients treated with new invented drugs enabling scientists to understand mechanism of action of drugs. Though 2D-gel electrophoresis and 2D-DIGE allow the whole protein expression of a cell to be investigated at a single point of time, they have weak point in detecting the significance of proteins during the process taking place over time. The future development of these 2 techniques for real time protein expression analysis will enhance the knowledge of how proteins behave in the real situations of cells. This may give the big impact in biomedical research development in order to study the pathology and improve the diagnosis and treatment of a variety of diseases.

Key words: 2D-gel electrophoresis, 2D-DIGE, proteomic, biomedical application

different conditions to accomplish the protein mapping of mouse tissues.[2] It was claimed that this technique was the powerful tool for the analysis and detection of proteins from complex biological samples. Recent advances have greatly increased as many as 10,000 proteins separated in a single gel.[3]

2. PRINCIPLE OF 2D-GEL ELECTROPHORESIS

In 2D-gel electrophoresis, proteins are separated according to isoelectric point (pI; pH value at which net charge of molecule equals to zero) in isoelectric focusing (IEF) gel as the first dimension and according to molecular weight in

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sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension[4,5] (Figure 1). For IEF gel, the immobilized pH gradient (IPG) polyacrylamide gels (pH 3-10) are prepared as gel strips and now available as commercial products. The scientists can select certain pH range appropriated to the sample of interest (i.e. pH 4-7 in Figure 1). When an electric field is applied, proteins start to migrate according to their charges toward the anode or the cathode, respectively, and stop migrating when their charge is neutral. After completion of first dimension separation, proteins are separated by molecular weight in SDS-PAGE. SDS is an anionic detergent which denatures the proteins, converting them to a linear molecule and a net negative charge. When combined with a reducing agent such as dithiothreitol (DTT), proteins are therefore separated exclusively by mass or molecular weights.

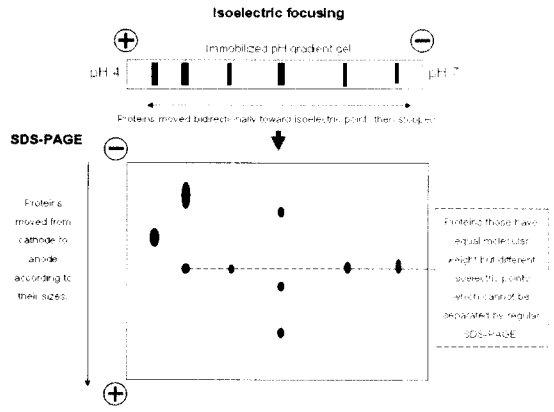

Figure 1 The principle of 2D-gel electrophoresis (modified from[5])

As people know, in one-dimensional SDS-PAGE, single band may contain one or more species of proteins which have the same molecular weight but different pI. But in 2D-gel electrophoresis, different proteins, even having the same molecular weight can be separated according to their different pI. So each spot on 2D-gel electrophoresis corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and the information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained.

To detect protein spots on 2D-gel, many methods can be used according to protein loading on gel (analytical or preparative), the purpose of the gel (protein quantitation or blotting), and the sensitivity required. The most common methods are silver staining[6] and Coomassie blue staining.[7] Other methods, including S^{35}-Met or C^{14} radiolabelling[8], colloidal gold[9], zinc imidazole[10], ponceau S, amido black[11], India ink[12], and fluorescent dye[13], can also be used in different applications to achieve better sensitivity.

After 2D-gel electrophoresis and protein visualization by staining, images of gels are digitized for computer analysis by image or fluorescent scanners, and are subjected to analyse by special image analysis software. The 2D-patterns are very complex and special software tools are required to find differentially expressed proteins, such as up or down-regulated proteins, post-translational modified proteins. Image spots on the gels are initially detected, manually edited and then processed by background subtraction and total spot volume normalization. The resulting spot percentage is used for comparison the level of proteins between different samples. The reliability of quantitative determinations of protein amounts in spots is largely dependent on the protein detection technique applied. Usually, only significantly up/

down-regulated spots or appearing/disappearing spots are selected for analysis with mass spectrometry to identify the species of protein, through sequence database searching.[14]

3. APPLICATIONS OF 2D-GEL ELECTROPHORESIS

A large and growing application of 2D-gel electrophoresis is PROTEOME analysis which is the analysis of entire PROTEin complement expressed by genOME.[15] Unlike the genome, there are potentially thousands of proteomes. Each cell type can alter its proteome depending on the unique tissue microenvironment in which it resides. Clinical proteomics, as a new and most exciting sub-discipline of proteomics, involve the bench-to-bedside clinical application of proteomic tools. The promise of clinical proteomics and related technologies, such as 2D-gel electrophoresis, is that diseases can be identified earlier through the discovery of biomarkers, that the next generation of drug targets can also be identified, and that we can then apply this knowledge to personalized medicine.[16] The ultimate goals of personalized medicine are to take advantage of a molecular understanding of disease, both to optimize drug development and direct preventive resources and therapeutic agents at individuals at risk while they are still well. This review aims to describe recent applications of 2D-gel electrophoresis in biomedical research. Such applications include detection of disease markers, monitoring therapies, vaccine development, drug study, and cancer research.

4. BIOMEDICAL APPLICATIONS OF 2D-GEL ELECTROPHORESIS

4.1 DETECTION OF DISEASE BIOMARKERS

For biomarker discovery in many important diseases, 2D-gel electrophoresis was used. The expressed protein profiles of hepatitis B virus (HBV)-infected patient sera were compared to those of normal sera aiming to search for disease-associated proteins that can be used as serological biomarkers for diagnosis and/or target proteins for pathological study.[17] At least 7 proteins were significantly changed in HBV-infected sera including haptoglobin B and A2 chain, apolipoprotein A (type I and IV), $\propto_1$-antitrypsin and DNA topoisomerase IIB. The changes displayed in both the quantity and expression pattern which the latter represented the changes of protein isoform in the HBV-infected patients. A combination simultaneously considering the quantities and isoforms of these proteins could be a useful serum biomarker or index for HBV diagnosis and therapy.

4.2 MONITORING THERAPIES

2D-gel electrophoresis was used to measure changes in synovial fluid proteins from patients with rheumatoid arthritis (RA).[18] RA is one of a number of autoimmune diseases in which T-lymphocytes are believed to be central to the etiology and pathogenesis.[19] The course of disease and the efficacy of antibody to CD_4 T-lymphocyte as the immunosuppressive agents were monitored by analyzing synovial fluid from the patients. The results indicated that changes in levels of some acute-phase proteins were correlated with the clinical improvement and serum C-reactive protein, a conventional clinical chemistry measurement.

4.3 VACCINE DEVELOPMENT

The other application of 2D-gel electrophoresis is the attempt to produce the effective vaccine against a variety of microbes and parasites.[20] A major obstacle to vaccine development is the difficulty to identify the antigens that mediated

protection from as many as 14,000 to 20,000 genes in the Schistosome genome. 2D-gel electrophoresis is used to resolve this problem. Soluble proteins can be separated in 2D-gel electrophoresis before western blotting to identify the full range of antigenic targets present in a parasite preparation. The next step is to discover which target proteins represent the weak points in the parasitic defenses. Then the most appropriated protein/glycoprotein can be used to induce in animal model to produce the effective antibody against the microbes and parasites in human. For example, 2D-gel electrophoresis was used to study the secreted proteins of the gastric pathogen, *Helicobactor pylori.* The result indicated the 33 separated secreted proteins.[21] Twenty-six of these were identified by mass spectrometry. Among these identified proteins, there were several interesting candidates for innovative approaches to treat or prevent *H. pyroli* infections. The oxidoreductases and serine protease might be interesting targets for antimicrobial agents that would interfere with the ability of *H. pyroli* to modify its microenvironment. Several secreted proteins are recognized by the host immune system, suggesting that they are interesting vaccine antigen candidates.

The other example is application of 2D-gel electrophoresis in *Opisthorchis viverrini* vaccine development. This parasite has been proven as the strong risk factor of cholangiocarcinoma in Southeast Asia, especially in the Northeastern part of Thailand.[22] Stimulation of immune response clearly occurs during both juvenile and adult stages through the release of antigens into the bile or host tissues.[23] Protein mixtures from different ages of parasites were analyzed in 2D-gel electrophoresis.[24] The total spots obtained from the fluke at 1, 2, 3 and 4 weeks old were 210, 221, 231 and 239, respectively. The profiles from 1, 2, 3 and 4 weeks old flukes were compared using the profile of 1 week old juvenile as a reference. All major differences between the 2D-gel profiles produced from these samples are highlighted by the use of boxes and circles in Figure 2. Proteins those are differentially expressed in the maturation process of the fluke are thought to be important for growth and development of the parasites, whereas those constantly expressed are thought to be essential for survival. These proposed proteins may be served as the target antigens associated not only with vaccine development against *O. viverrini,* but also with pathogenesis of cholangiocarcinoma in the future.

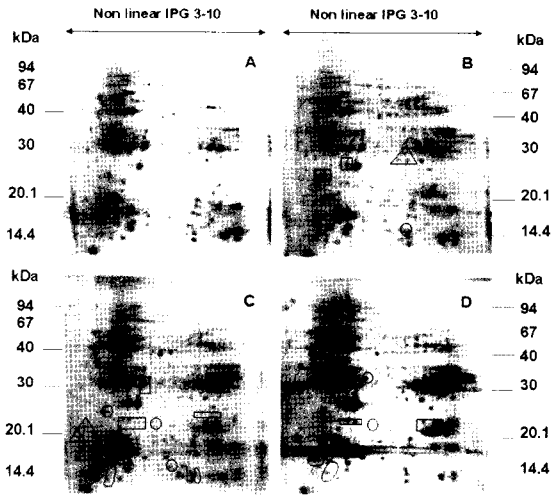

Figure 2 2D-gel electrophoresis of a total homogenate of *O. viverrini* at different ages: (A) 1 week juvenile, and B, C and D are flukes at 2, 3 and 4 weeks post infection, respectively.[24] (Printed with permission)

4.4 DRUG STUDY

To study the mechanisms of drug actions, 2D-gel electrophoresis and mass spectrometry were used for the identification of compound specific markers in rat livers induced by various agents including hepatotoxicants, methapyrilene and dexamethaxone.[25] Many changes in proteins could be associated with these known pharmacological and toxicological mechanisms of actions of these drugs. Another application of 2D-gel electrophoresis in studying of drug action is to study the increased plasma protein excretion in urine as the consequence of nephropathy and nephritic syndrome induced by puromycin aminonucleoside in rats.[26]

4.5 CANCER RESEARCH

There is intense interest in applying proteomic technologies to uncover processes involved in neoplastic transformation and new biomarkers that correlate with early diagnosis, as well as to accelerate the development of new therapeutic targets and clues to understand the molecular characterization of cancer progression.[27,28] The search of new proteins by proteomic method is a major goal in tumor biology and may lead to the detection of markers or antigens for the generation of tumor vaccines. Recently, 10 tumor cell lines were used in 2D-gel electrophoresis with subsequent in-gel digestion and protein identification.[29] A series of 10 proteins were detected and three of them observed in lung cancer and malignant melanoma which might be the candidates for development of tumor markers and generation of tumor vaccines. The same procedure was used to show the expressed proteins in pancreatic adenocarcinoma compared to normal pancreatic tissues.[30] These newly identified may eventually serve as diagnostic markers or therapeutic agents.

5. 2D-DIFFERENTIAL IN-GEL ELECTROPHORESIS (2D-DIGE)

Although 2D-gel electrophoresis has been widely used and successfully applied in a variety of biological systems, several technical limitations still exist. It typically requires some computerized justification of 2D-gel images so that two images can be superimposed and compared. These difficulties limit the speed and accuracy of quantitation of protein spots in 2D-gel electrophoresis. The differential in-gel electrophoresis (DIGE) technique has been recently introduced and aimed at improving reproducibility and accuracy. The concept of DIGE was developed by Minden and colleagues.[31] Each sample has been labeled with different fluorescent cyamine dyes (Cy2, Cy3, Cy5) before 2D-gel electrophoresis in the same gel. Normally, a control sample is labeled with Cy3 and diseased sample with Cy5 or vice versa. The pooled samples (equal amount of control and diseased samples) is created and labeled with the third dye, Cy2. The pooled sample is used as an internal standard that will confirm actual protein changes or experimental artifacts. Each dye can be visualized under a different wavelength and the images overlayed, giving a combined image that can be analyzed using various software packages.[32] This technique allows direct comparison between samples to show the presence of a particular protein, for instance, in a tested sample compared with control, reducing the effect of inter-gel variation and minimizing the reproducibility problem.[33] In conclusion, this effectively removes system variation enabling accurate quantitation of real

protein differences between samples.

6. APPLICATIONS OF 2D-DIGE

6.1 STUDY OF DRUG ACTION

The application of 2D-DIGE was found in the investigation of the mechanism of action of a new invented anti-tumor drug (Figure 3).[34] Briefly, in concept, to gain insight into the agent's biological activity, the interaction proteins from cell lysate prepared from human cancer cell line with active anti-tumor agent were collected from passing the cell lysate through the drug affinity chromatography. To control the nonspecific binding, the affinity chromatography with the inactive drug (drug analogue), was performed in parallel. The eluants from the two columns were compared using 2D-DIGE to identify the proteins enriched in the active column compared with the inactive column. Spots found to be significantly increased in the active sample were excised and identified by mass spectrometry. This approach has enabled the identification of proteins that bind specifically to a drug molecule but not to an inactive analogue. Consequently, the identified protein can now be investigated further to gain more insights into the *in vivo* mechanism of action of that anti-tumor drug.

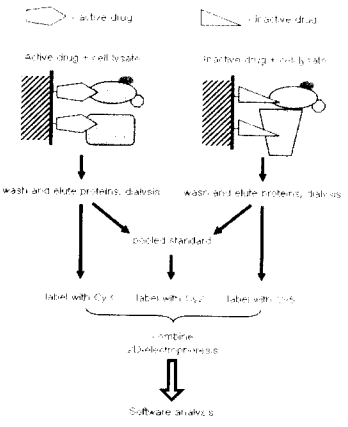

Figure 3 2D-DIGE in drug action study (modified from[34])

6.2 CANCER RESEARCH

The esophageal cancer-specific protein markers were discovered by the advantage of 2D-DIGE.[35] The result identified 1,038 protein spots in cancer cell lysates and 1,088 protein spots in normal cell lysates. Of the detected proteins, 58 spots were up-regulated and 107 were down-regulated in cancer cells. As mentioned earlier, global quantification of protein expression between cancer and normal cells in combination with mass spectrometry is a powerful tool for the molecular characterization of cancer progression and identification of cancer-specific protein markers. Moreover, proteomic analysis of a human cancer employing the 2D-DIGE technique highlights several advantages. First, because the two pools of protein extracts were separated in the same gel, the reproducibility compared with conventional 2D-gel separation is improved, and the comparison of protein expression patterns is simplified. Second, the differences in protein expression between two samples can be easily identified, based on fluorescence of labeled Cy3 and Cy5 dyes, thus providing accurate quantitation of protein changes.

7. FUTURE PROSPECT IN 2D-GEL ELECTROPHORESIS

2D-gel electrophoresis is the important technique classified as proteomic technology. Although the resolving power of 2D-gel electrophoresis remains unchallenged, mass spectrometry has become more sensitive, faster and more reproducible. However, examination of whole protein expression of cell or organism by these 2 current proteomic technologies is like taking a snapshot of its activity at a single point of time. This may underestimate or miss the significance

of processes taking place over time. The future proteomic technology will focus on real-time proteomics, i.e. monitoring the proteome in a real time/time-lapse manner. This greatly enhances the knowledge of how protein behaves at certain situation in a cell.

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